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Calcium transport in membrane vesicles of *Streptococcus cremoris*

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Rightside-out membrane vesicles of *Streptococcus cremoris* were fused with proteoliposomes containing the light-driven proton pump bacteriorhodopsin by a low-pH fusion procedure reported earlier [Driessen, A. J. M., Hellingwerf, K. J. & Konings, W. N. (1985) *Biochim. Biophys. Acta 808*, 1–12]. In these fused membranes a proton motive force, interior positive and acid, can be generated in the light and this proton motive force can drive the uptake of Ca^{2+}. Collapsing $\Delta\psi$ with a concomitant increase in ΔpH stimulates Ca^{2+} uptake while dissipation of the ΔpH results in a reduced rate of Ca^{2+} uptake. Also an artificially generated ΔpH, interior acid, can drive Ca^{2+} uptake in *S. cremoris* membrane vesicles.

Ca^{2+} uptake depends strongly on the presence of external phosphate while Ca^{2+}-efflux-induced proton flux is independent of the presence of external phosphate. Ca^{2+} accumulation is abolished by the divalent cation ionophore A23187.

Calcium extrusion from intact cells is accelerated by lactose. Collapse of the proton motive force by the uncoupler carbonylcyanide *p*-trifluoromethoxyphenylhydrazone or inhibition of the membrane-bound ATPase by *N*,*N'*-dicyclohexylcarbodiimide strongly inhibits Ca^{2+} release. Further studies on Ca^{2+} efflux at different external pH values in the presence of either valinomycin or nigericin suggested that Ca^{2+} exit from intact cells is an electrogenic process. It is concluded that Ca^{2+} efflux in *S. cremoris* is mediated by a secondary transport system catalyzing exchange of calcium ions and protons.

Calcium ions play an important role as regulators of a large number of cellular processes in eukaryotes. The intracellular Ca^{2+} ion concentration is usually low as a result of energy-dependent Ca^{2+} influx and efflux processes. In prokaryotes a role of Ca^{2+} in a number of cellular functions and processes has been demonstrated or suggested [1]. Bacteria keep their intracellular Ca^{2+} concentration well below that of the surrounding medium as long as metabolic energy is available [1, 2].

Ca^{2+} can enter the bacterial cells either via a uniport system [3–5] or electrophoretically by diffusion through the membrane [6]. In most bacteria Ca^{2+} extrusion is catalyzed by a calcium/cation exchange system with protons as the most common cation [3, 5, 7–11]. However, in some bacteria living in extreme environments Ca^{2+} extrusion coupled to the sodium gradient has been found [12, 13]. Recently, a second Ca^{2+} extrusion system in *Escherichia coli* has been described that catalyzes cotransport of Ca^{2+} and phosphate [8]. In *Streptococcus faecalis* [6] and in the cyanobacterium *Anabaena variabilis* [14] Ca^{2+} is extruded by a primary Ca^{2+} pump which has been shown to be sensitive to vanadate [14–16]. The Ca^{2+} ATPase of *S. faecalis* has been reconstituted into liposomes from detergent-solubilized membranes [15, 17].

By using a novel system for generating a proton motive force in membrane vesicles from *Streptococcus cremoris*, we were able to demonstrate proton motive force (Δp) linked Ca^{2+} [18]. *S. cremoris* membrane vesicles were fused with proteoliposomes containing the light-induced proton pump bacteriorhodopsin (bR) by a low-pH fusion procedure [19]. The orientation of bR in the fused membranes was such that upon illumination an inside-out oriented Δp, interior positive and acid, was generated. This Δp functions as a driving force for the accumulation of Ca^{2+}, most likely via a Ca^{2+}/H^+ exchange system [18]. This activity is distinctively different from that described for *S. faecalis* [6]. In this report the properties of this Ca^{2+} transport system were analyzed in *S. cremoris* membrane vesicles either energized by artificial pH gradients or by light using bR as a Δp generating system.

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Abbreviations. bR, bacteriorhodopsin; Ph_4P^+, tetraphenyl phosphonium; Ph_4B^-, tetraphenyl boron; diS-C_3-(5), 3',3'-dipropylthiadicarbocyanine; Δp, proton motive force; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane proton gradient; $(cHxN)_2C$, *N*,*N'*-dicyclohexylcarbodiimide; $CF_3OPhzC(CN)_2$, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; S-13, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide; $ClHgPhSO_3$, *p*-chloromercuriphenylsulphonate; Ptd_2Gro, diphosphatidylglycerol (cardiolipin).

MATERIALS AND METHODS

Growth of cells and isolation of membrane vesicles

Streptococcus cremoris Wg2 (prt^-) was grown anaerobically on MRS broth at a controlled pH of 6.4 in a 5-l fermenter. Membrane vesicles of *S. cremoris* were prepared as described previously [20] suspended in 50 mM potassium phosphate pH 7.0 containing 10 mM $MgSO_4$ at a concentration of 10–15 mg protein/ml and stored in liquid nitrogen.

Halobacterium halobium NRL (strain R7 [21]) was grown according to the method described by Danon and Stoeckenius [22]. Purple membrane sheets were isolated as described [18] and resuspended in distilled water to a protein concentration of about 12 mg/ml and stored in liquid nitrogen.

Incorporation of bacteriorhodopsin in liposomes

Purple membranes were resuspended in 50 mM potassium phosphate, supplemented with 0.1 mM EDTA to a protein concentration of 4 mg/ml. Cardiolipin was added to the purple membrane suspension to a final concentration of 20 mg/ml. The suspension was sonicated using a 9-mm probe sonicator, at a frequency of 21 kHz and an amplitude of 4 μm (peak to peak) under nitrogen atmosphere for a total of 700 s at 4°C. Alternating intervals of 15-s sonication and 45-s rest were used. bR Liposomes were concentrated ($150000 \times g$; 60 min; 4°C) and used immediately after preparation.

Fusion between S. cremoris *membrane vesicles and bR proteoliposomes*

Fusion was induced using the low-pH fusion technique [18, 19]. *S. cremoris* membrane vesicles (2 mg protein) and bR proteoliposomes, containing 150 nmol bR, were mixed in a final volume of 1 ml. The pH of the magnetically stirred suspension was lowered to 5.0 by adding small aliquots of 1 M HCl. After 15 min at 25°C, the pH was readjusted to pH 7.0 with 1 M KOH. The fused membranes were vortexed and collected by centrifugation ($48000 \times g$; 30 min; 4°C). Membranes were washed once in 50 mM potassium phosphate pH 7.0.

Measurement of the proton motive force

The $\Delta\psi$ (interior positive) was calculated from the distribution of tetraphenylboron (Ph_4B^-) across the membrane using a Ph_4B^- selective electrode [23]. Reaction mixtures contained (final concentration) 10 mM sodium phosphate (pH 7.5), 50 mM NaCl, 1 μM Ph_4B^-, 2 mM $MgCl_2$, 0.1 μM tetraphenylphosphonium (Ph_4P^+), membranes containing 0.2 mg *S. cremoris* membrane protein, and 15 nmol bR. The accumulation of Ph_4B^- was calculated from the amount of Ph_4B^- which disappeared from the external medium. A correction for concentration-dependent binding of Ph_4B^- to the membranes, according to the model of Lolkema et al. [24], was applied. The pH gradient (ΔpH, interior acid) was estimated from the fluorescence quenching of 9-aminoacridine [25]. Reaction mixtures contained (final concentration) 10 mM sodium phosphate (pH 7.5), 50 mM NaCl, 5 μM 9-aminoacridine, 2 mM $MgCl_2$ and membranes as indicated above. 9-Aminoacridine was excited at 405 nm and the emitted light was monitored at 455 nm. Fluorescence quenching of 9-aminoacridine was calibrated with an artificially imposed ΔpH, interior acid. The external pH was changed by the addition of small aliquots of 1 M NaOH. In the presence of 4 μM nonactin the decay of the artificially imposed ΔpH could be fitted by a single exponent. Imposition of ΔpH values of different magnitudes and extrapolating to zero time resulted in a calibration curve from which the magnitude of the ΔpH, generated by bR upon illumination, could be estimated.

Internal pH change in *S. cremoris* membrane vesicles were determined by the use of entrapped pyranine [26]. *S. cremoris* membrane vesicles (5 mg/ml) were rapidly frozen in liquid nitrogen in the presence of 100 μM pyranine. Frozen membranes were thawed at room temperature for 20 min and sonicated for 50 s in a bath-type sonicator (Sonicor 50 W, Sonicor Instruments Corporation, New York). External pyranine was removed by chromatography on Sephadex G-50 (1×16 cm) and the pyranine-loaded membranes were recovered in the void volume of the column. Pyranine fluorescence was measured at excitation and emission wavelengths of 460 nm and 508 nm, respectively.

Artificially imposed $\Delta\psi$, interior negative, was recorded using the fluorescence quenching of diS-C_3-(5) [27]. Membrane vesicles (0.1 mg protein) prepared in 50 mM potassium phosphate (pH 7.0) were diluted 100-fold into 50 mM sodium phosphate (pH 7.0) supplemented with 10 mM $MgSO_4$ and 2 μM diS-C_3-(5). Subsequently valinomycin was added to the suspension (2 nmol valinomycin/mg protein). Fluorescence of diS-C_3-(5) was measured at 670 nm using an excitation wavelength of 622 nm.

Transport assays

Uptake studies of calcium and methylamine were performed by filtration as described [28]. Fused membranes were resuspended into 100 μl 50 mM potassium phosphate, pH 7.5 (unless indicated otherwise), containing 2 mM $MgCl_2$. The suspension was stirred and illuminated with white light with an intensity of about 1 kJ/m^2 · s for 2 min prior to the addition of 200 μM $^{45}CaCl_2$. Other additions are indicated in the legends to figures. At the times indicated uptake was terminated by addition of 2.0 ml ice-cold 0.1 M LiCl and filtered over cellulose acetate filters with 0.45-μm pore size, which were presoaked in 0.2 mM $CaCl_2$ to reduce nonspecific binding of Ca^{2+} to the filters. The final protein concentrations in all transport experiments were between 1 – 2 mg/ml.

For calcium uptake energized by an artificially imposed ΔpH, interior acid, nigericin was added to 0.1 ml of a concentrated membrane vesicle suspension (25 mg membrane protein/ml) to a final concentration of 0.2 nmol/mg protein. After incubation on ice for 30 min, samples of 1 μl were rapidly diluted into 100 μl 50 mM choline phosphate (pH 7.0) containing 2 mM $MgCl_2$ and 500 μM $^{45}CaCl_2$ or 40 μM [^{14}C]methylamine. Uptake experiments were performed as described above. The final nigericin concentration did not exceed 0.5 μM. An artificial $\Delta\psi$, interior positive, was imposed by washing membrane vesicles three times in 50 mM sodium phosphate (pH 7.0) containing 2 mM $MgCl_2$ in the presence of 2 nmol valinomycin/mg protein. The concentrated membrane suspension (1 μl) was subsequently diluted into 100 μl 50 mM potassium phosphate (pH 7.0) containing 2 mM $MgCl_2$ and 500 μM $^{45}CaCl_2$. An artificial $\Delta\psi$, interior negative, was imposed by diluting potassium loaded membrane vesicles into 50 mM sodium phosphate (pH 7.0) essentially as described for an artificial $\Delta\psi$, interior positive.

For calcium efflux studies membrane vesicles or intact cells (late log phase) were incubated for 3 h at 4°C, with 0.2 – 1.0 mM $^{40}Ca^{2+}$ or $^{45}Ca^{2+}$. Small aliquots were subsequently diluted 100-fold into a Ca^{2+}-free buffer and the release of $^{45}Ca^{2+}$ or uptake of [^{14}C]methylamine was assayed as described above.

All experiments were done at 25°C. Radioactivity was determined with a liquid scintillation counter. The internal concentration of solutes accumulated by the vesicles was calculated using a value for the intravesicular volume of 4.3 μl/mg protein [20].

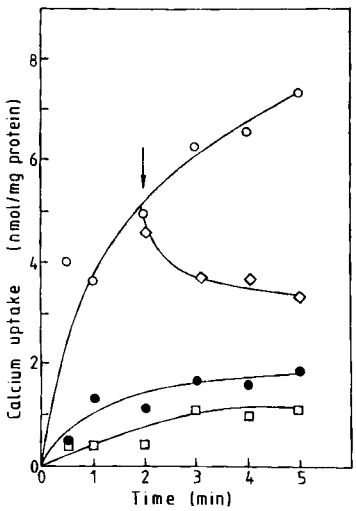

Fig. 1. *Ca^{2+} uptake by* S. cremoris *membrane vesicles fused with bacteriorhodopsin proteoliposomes in the light (○), in the dark (●) and in the light with the uncoupler $CF_3OPhzC(CN)_2$ (10 μM) (□).* At the arrow A23187 (30 μM) (◇) was added. Experiments were performed in the presence of 2 mM $MgCl_2$

Protein and phospholipid determination

Protein was measured by the method of Lowry et al. [29], using bovin serum albumin as standard. Phospholipid concentrations were determined by analysis of lipid phosphorous [30]. The concentration of purple membranes was determined using a molar absorption coefficient at 560 nm of 63000 M^{-1} cm^{-1} [31].

Materials

[^{14}C]Methylamine (2.22 TBq/mmol) and [^{45}Ca]$CaCl_2$ (1.85 TBq/mmol) were obtained from Amersham International (Amersham, Bucks, UK). Beef-heart cardiolipin was from Sigma. diS-C_3-(5) was obtained from Molecular Probes, Inc. (Junction City, OR). Ionophores and uncouplers were dissolved in pure ethanol. Additions of these compounds were made so that the final ethanol concentration in the transport assay did not exceed 1% (v/v). All other chemicals were reagent grade and obtained from commercial sources.

RESULTS

Light-dependent uptake of calcium by S. cremoris *membrane vesicles fused with bacteriorhodopsin proteoliposomes*

Illumination of *S. cremoris* membrane vesicles fused with bacteriorhodopsin (bR) proteoliposomes by low-pH treatment resulted in a significant accumulation of Ca^{2+} (Fig. 1). Accumulation of Ca^{2+} is demonstrated by the action of the ionophore A23187, which mediates the electroneutral exchange of divalent cations for protons. Addition of this ionophore (Fig. 1, arrow) caused release of accumulated Ca^{2+} when Mg^{2+} was present externally. In the absence of Mg^{2+}, A23187 stimulated Ca^{2+} uptake. This phenomenon was also observed in bR proteoliposomes (data not shown) and is consistent with the known ability of A23187 to mediate Ca^{2+}/Mg^{2+} exchange. Uptake of Ca^{2+} was dependent on a Δp generated by bR in the presence of light since only binding of Ca^{2+} to the membranes was observed when the experiments were performed in the dark or in the light in the presence of the uncoupler carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone, $CF_3OPhzC(CN)_2$ (Fig. 1).

Table 1. *The Δp and the initial rate of Ca^{2+} uptake in* S. cremoris *membrane vesicles fused with bacteriorhodopsin proteoliposomes upon illumination*

Experiments were performed in 10 mM sodium phosphate (pH 7.5) supplemented with 50 mM NaCl and 2 mM $MgCl_2$. $V_{Ca^{2+}}$ is the initial Ca^{2+} uptake rate; the relative rate is indicated in parentheses. Relative rates are expressed as percentages of the rate observed without ionophores minus the rate observed in the presence of 10 μM S-13

Additions	$-Z\Delta pH$	$\Delta\psi$	Δp	$v_{Ca^{2+}}$
	mV			nmol·mg protein^{-1} min^{-1} (%)
None	+32	+14	+46	4.8 (100)
Triphenyltin (40 nM)	0	+51	+51	1.9 (9)
Nonactin (4 μM)	+42	0	+42	6.4 (150)
S-13 (10 μM)	0	0	0	1.6 (0)

Information about the driving force for Ca^{2+} uptake was obtained by studying the effects of ionophores on the Δp and the initial Ca^{2+} uptake rate (Table 1). These experiments were performed in a potassium-free buffer since the $\Delta\psi$ probe tetraphenylboron (Ph_4B^-), which is accumulated in response to a $\Delta\psi$, interior positive, precipitated in the presence of potassium. Uptake of Ca^{2+} was stimulated by nonactin, which dissipates the $\Delta\psi$ by the electrogenic influx of Na^+. This collapse of the $\Delta\psi$ was compensated by an increase of the ΔpH. In the presence of triphenyltin, which collapses the ΔpH by an electroneutral exchange of chloride ions for hydroxyl ions [32] hardly any uptake of Ca^{2+} was observed. Since the ΔpH was compensated by an increase of the $\Delta\psi$, the results indicate that the ΔpH functions as the major driving force for Ca^{2+} uptake under the conditions employed.

Artificial ΔpH dependent uptake of calcium by S. cremoris *membrane vesicles*

More direct information about the involvement of the ΔpH in Ca^{2+} transport can be obtained by the imposition of an artificial proton gradient across the vesicular membrane. For these experiments membrane vesicles were loaded with potassium in the presence of nigericin, an electroneutral exchanger of potassium for protons. Subsequent dilution of these vesicles into choline-containing buffer (i.e. potassium-free buffer), thereby imposing an outwardly directed potassium diffusion gradient, resulted in a transient acidification of the vesicle interior. This is demonstrated by the accumulation of the weak base methylamine (Fig. 2B). This ΔpH, interior acid, could drive the uptake of Ca^{2+} (Fig. 2A). When choline was replaced by sodium the Ca^{2+} uptake was lower (Fig. 2A), but also the extent of acidification was reduced under these conditions (Fig. 2B). Imposition of a ΔpH by a 10-fold dilution (theoretically leading to a ΔpH of 1) resulted in an internal acidification of 0.6 pH difference in choline buffer and of only 0.25 pH difference in sodium buffer as measured with the fluorescent pH probe pyranine [26] entrapped in the membrane vesicle interior (data not shown). These results can be explained by the known ability of nigericin to mediate not only potassium/proton exchange but also, albeit at a lower rate, sodium/proton exchange. Dilution of the membrane vesicles into potassium buffer did not result

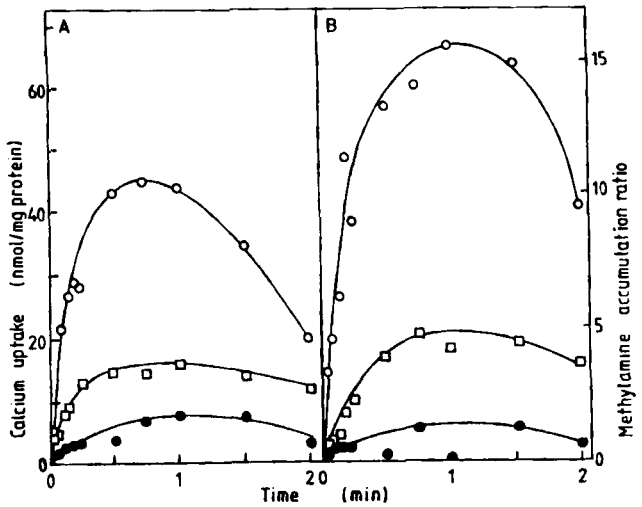

Fig. 2. *Ca^{2+} (A) and methylamine (B) uptake by* S. cremoris *membrane vesicles upon imposition of a nigericin-mediated ΔpH.* Potassium-loaded membrane vesicles were diluted at pH 7.0 into 50 mM choline phosphate (○), 50 mM sodium phosphate (□) and 50 mM potassium phosphate (●) supplemented with 2 mM $MgCl_2$

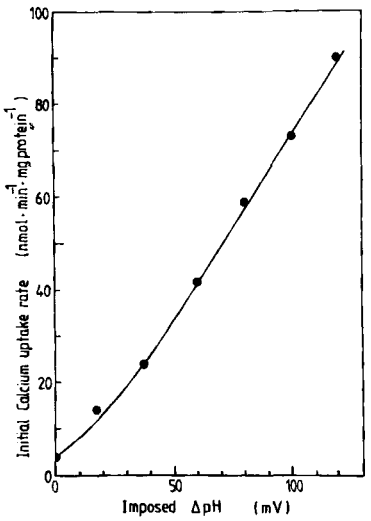

Fig. 3. *Relation between the ΔpH, interior acid, and the initial rate of Ca^{2+} uptake by* S. cremoris *membrane vesicles*

in the generation of a ΔpH nor in Ca^{2+} uptake (Fig. 2). Ca^{2+} uptake was also not observed when the dilution of the vesicles into choline buffer was performed in the presence of the uncoupler $CF_3OPhzC(CN)_2$ (10 μM) (data not shown).

The magnitude of the ΔpH was varied by changing the external potassium concentration. The rate of Ca^{2+} uptake increased with the ΔpH (Fig. 3). It should be emphasized that at large imposed ΔpH the measured ΔpH did not correlate linearly with the theoretically imposed ΔpH (data not shown). A non-linear relation between the driving force (ΔpH) and the initial rate of Ca^{2+} uptake could be the result of this observed discrepancy. Upon imposition of a nigericin-mediated ΔpH, interior acid, a $\Delta\psi$, interior negative, could be generated by electrogenic fluxes of potassium via nigericin dimers [33]. Therefore, the uptake of Ca^{2+} in response to a ΔpH created by external pH changes was studied. Dilution of membrane vesicles with an internal pH of 6.0 into a buffer of pH 8.0 resulted in a transient accumulation of Ca^{2+}. In

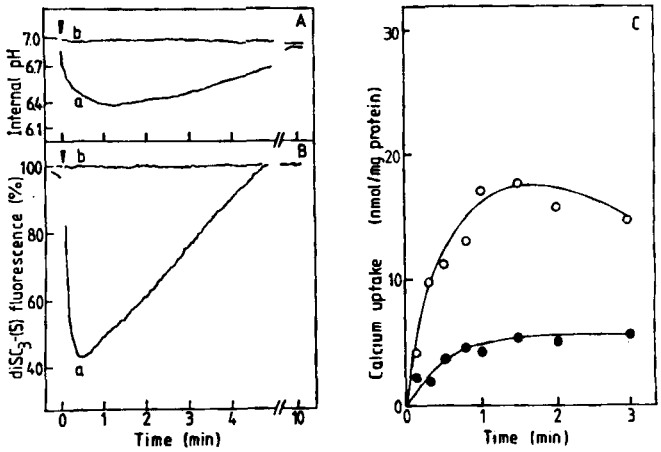

Fig. 4. *Effect of an artificially imposed $\Delta\psi$, interior negative, on fluxes of protons across the membrane (A), diS-C_3-(5) fluorescence quenching (B) and Ca^{2+} uptake (C).* The internal pH change was monitored by the fluorescence of vesicle-entrapped pyranine and calibrated by the addition of KOH/HCl in the presence of nigericin. Curve (a) was obtained when potassium-loaded membrane vesicles were diluted into 50 mM sodium phosphate in the presence of valinomycin; curve (b) was obtained upon dilution of the potassium-loaded membrane vesicles into 50 mM potassium phosphate. Ca^{2+} uptake (C) was in the presence (○) or absence (●) of a potassium gradient

control experiments membrane vesicles were incubated in the alkaline buffer for 15 min prior to the addition of Ca^{2+} in order to allow the pH gradient to dissipate. Under these conditions no Ca^{2+} uptake was observed (data not shown).

The effects of a $\Delta\psi$, interior positive, on Ca^{2+} uptake were studied by imposing an inwardly directed potassium diffusion gradient in the presence of valinomycin by diluting sodium-loaded membrane vesicles into potassium-containing buffer. Under these conditions no uptake of Ca^{2+} was observed. In contrast, significant levels of Ca^{2+} uptake were observed when a $\Delta\psi$, interior negative, was imposed (Fig. 4C). This was achieved by diluting potassium-loaded membrane vesicles into sodium-containing buffer in the presence of valinomycin. Uptake of Ca^{2+} under these conditions could occur as an electrophoretic diffusion of Ca^{2+} in response to a $\Delta\psi$, interior negative. However, since the uptake of Ca^{2+} does not follow the time course of the $\Delta\psi$ (as monitored by the quenching of diS-C_3-(5) fluorescence, Fig. 4B, curve a) it is more likely a result of the ΔpH, interior acid (Fig. 4A, curve a) generated as a result of a $\Delta\psi$-induced proton flux across the membrane [34] (and A. J. M. Driessen, unpublished results). This everted ΔpH was not abolished by $(cHxN)_2C$ which blocks the F_0 part of the F_1F_0 ATPase (data not shown).

Effects of various reagents on Ca^{2+} transport

Uptake of Ca^{2+} was only observed when phosphate was present externally. Maximal stimulation of energy-dependent Ca^{2+} uptake was observed in the presence of 15 mM phosphate (Fig. 5). Energy-independent binding of Ca^{2+} increased with increasing external phosphate concentration up to 100 mM.

Chelation of external phosphate with 10 mM ammonium molybdate [35] resulted in a rapid release of accumulated Ca^{2+} (Fig. 6). In the presence of external Mg^{2+} (2 mM), addition of EDTA (1 mM) also caused a rapid release and the

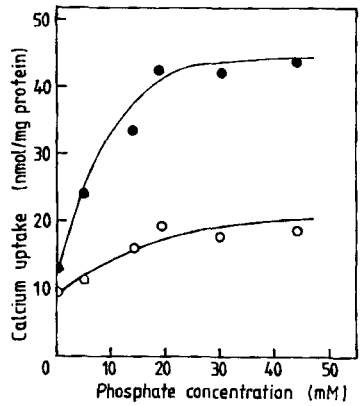

Fig. 5. *Effect of the external phosphate concentration on the energy-dependent (●) and energy-independent (○) maximal level Ca^{2+} uptake.* Experiments were performed in 10 mM K^+/Hepes (pH 7.0) supplemented with 100 mM KCl. Instead of KCl, increasing amounts of potassium phosphate (pH 7.0) were included in the buffer. Further experimental conditions were as described in the legend of Fig. 2 and in Materials and Methods

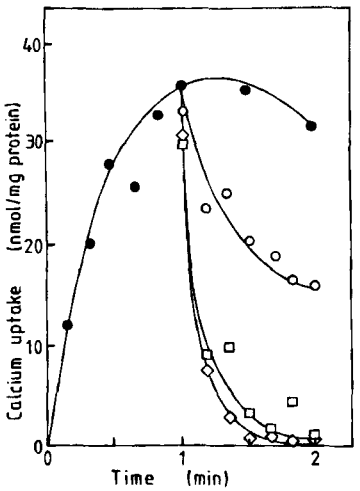

Fig. 6. *Effect of (○) the divalent cation ionophore A23187 (30 μM), (□) EDTA (1 mM) and (◇) ammonium molybdate (10 mM) on (●) ΔpH, interior acid, driven Ca^{2+} uptake by membrane vesicles of* S. cremoris. Experimental conditions were as described in the legends to Fig. 2 and in Materials and Methods

addition of the divalent cation ionophore A23187 resulted in a slow release of accumulated Ca^{2+}. In the presence of 10 mM Mg^{2+}, Ca^{2+} release by the ionophore proceeded more rapidly (data not shown).

La^{3+} is not a very potent inhibitor of Ca^{2+} uptake (Table 2). At high La^{3+} concentrations (>40 μM) extensive aggregation of membrane vesicles occurred which precluded further use of this inhibitor. Ruthenium red, an inhibitor of several eukaryotic Ca^{2+} extrusion systems had a more pronounced effect on Ca^{2+} uptake. Ca^{2+} uptake was not sensitive to trypsin treatment of the membrane vesicles and *N,N'*-dicyclohexylcarbodiimide [$(cHxN)_2C$], whereas 1 mM *p*-chloromercuriphenylsulphonate ($ClHgPhSO_3$) almost completely inhibited Ca^{2+} uptake. Inactivation by $ClHgPhSO_3$ was largely reversed by dithiothreitol.

Ca^{2+} efflux-induced proton fluxes

As a result of the relatively high rate of Ca^{2+} uptake, significant fluxes of protons upon Ca^{2+} efflux from membrane vesicles could be measured. Membrane vesicles were passively loaded with 1 mM Ca^{2+} by incubation at 25°C for 60 min. Dilution of a concentrated suspension of Ca^{2+}-loaded membrane vesicles (18 mg protein/ml) into a Ca^{2+}-free buffer resulted in a transient uptake of the weak base methylamine (Fig. 7). In parallel experiments quenching of 9-aminoacridine was observed (data not shown). In the presence of nigericin (0.2 μM) or externally added Ca^{2+} (1 mM) the uptake of methylamine was significantly reduced. Acidification of the membrane vesicle interior was also observed in potassium/Hepes when external phosphate was absent. This acidification was not affected by valinomycin.

Increasing concentrations of Mg^{2+} inhibited acidification progressively and 50% inhibition was observed in the presence

Table 2. *Effects of various reagents on the initial rate of Ca^{2+} uptake by* S. cremoris *membrane vesicles upon imposition of a nigericin-mediated potassium diffusion gradient, leading into a ΔpH, interior acid*
Potassium-loaded membrane vesicles (25 mg protein/ml), pre-incubated with the compound indicated, were diluted into 50 mM choline phosphate (pH 7.0), supplemented with 2 mM $MgCl_2$ as described under Materials and Methods. Activity was determined from the uptake of Ca^{2+} after 10 s

Addition	Concentration	Activity
	μM	%
None	–	100
$CF_3OPhzC(CN)_2$	10	23.7
S-13	5	20.3
A23187 + 10 mM $MgCl_2$	20	30.0
$(cHxN)_2C$	50	95.0
Ruthenium red	10	48.2
	20	36.2
$LaCl_3$	10	85.5
	20	68.0
Trypsin	(10)[a]	93.0
$ClHgPhSO_3$	1000	24.5

[a] 10 μg trypsin/mg protein for 10 min at 30°C.

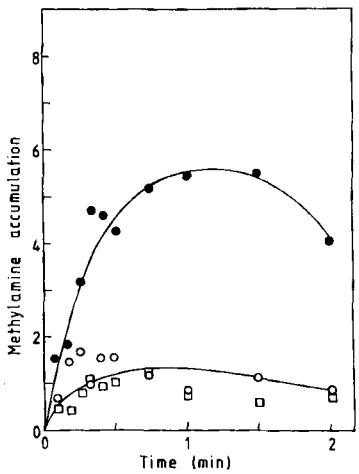

Fig. 7. *Ca^{2+} efflux-induced uptake of methylamine in membrane vesicles of* S. cremoris. Ca^{2+}-loaded membrane vesicles were diluted 100-fold into a Ca^{2+}-free buffer (●), in the presence of 1 μM nigericin (□) or into a buffer containing 1 mM Ca^{2+} (○). Experiments were performed in 10 mM K^+/Hepes (pH 7.0) supplemented with 100 mM KCl as described in Materials and Methods

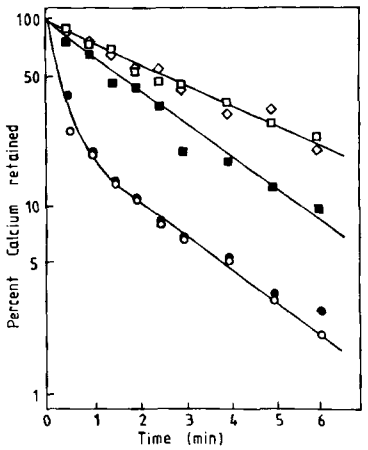

Fig. 8. *Ca^{2+} efflux from intact cells of* S. cremoris. Ca^{2+} efflux in the absence of externally added energy source (■) and in the presence of lactose (6 mM) (○). The effects of (●) vanadate (300 μM), (□) $(cHxN)_2C$ (50 μM) and (◇) $CF_3OPhzC\text{-}(CN)_2$ (10 μM) were studied in the presence of lactose (6 mM). Ca^{2+}-loaded cells (1 mM Ca^{2+} to 80 mg dry weight/ml) were diluted 100-fold in 50 mM potassium phosphate (pH 7.0) supplemented with 2 mM $MgCl_2$

of 10 mM $MgCl_2$ (data not shown). Acidification was completely inhibited by 1 mM $MnCl_2$. Similar observations were done using intact cells (data not shown).

Energetics of Ca^{2+} efflux from intact cells

Energy-depleted cells were loaded with $^{45}Ca^{2+}$ (1 mM) at 4 °C for 3 h by passive equilibration. When the Ca^{2+}-loaded cells were diluted into a Ca^{2+}-free buffer a slow release of Ca^{2+} was observed. The efflux of Ca^{2+} was increased by the presence of the energy source lactose (Fig. 8). Under these conditions both a proton motive force and ATP is generated. This Ca^{2+} efflux was strongly inhibited by the F_1F_0 ATPase inhibitor $(cHxN)_2C$ (50 μM) and by the uncoupler $CF_3OPhzC(CN)_2$ (10 μM), indicating the role of the proton motive force in Ca^{2+} release. Orthovanadate, an inhibitor of Ca^{2+} extrusion in intact cells of *S. faecalis* [16], had no effect on Ca^{2+} efflux up to a concentration of 300 μM. The effects of the ionophores nigericin and valinomycin on energy-dependent Ca^{2+} extrusion were studied at different external pH values (Table 3). At low external pH (5.5) nigericin almost completely inhibited Ca^{2+} release while valinomycin stimulated the rate of Ca^{2+} efflux. At higher pH values valinomycin became more inhibitory. Although the absolute initial rates varied between different experiments the general effects of the ionophores remained the same.

Table 3. *Effects of valinomycin and nigericin on the energy-dependent efflux of Ca^{2+} from intact cells of* S. cremoris *at different external pH values*

$v_{Ca^{2+}}$ is the initial rate of calcium uptake, determined from the amount of $^{45}Ca^{2+}$ released in 2 min. Percentage inhibition or stimulation is indicated in parentheses. Relative activities are expressed as percentages of the rate observed without addition minus the rate observed in the presence of $(cHxN)_2C$ at the pH indicated. Cells were loaded with Ca^{2+} as described under Materials and Methods and diluted 100-fold in Ca^{2+}-free buffer containing 50 mM potassium phosphate, 2 mM $MgSO_4$ and 6 mM lactose

Additions	$v_{Ca^{2+}}$ at external pH		
	5.5	6.5	7.5
	nmol · mg protein^{-1} · min^{-1} (%)		
None	4.0 (100)	13.8 (100)	8.6 (100)
$(cHxN)_2C$ (100 μM)	1.4 (0)	7.4 (0)	4.3 (0)
Nigericin (1 μM)	1.9 (17)	9.6 (33)	7.1 (66)
Valinomycin (10 μM)	6.1 (180)	10.9 (53)	4.5 (5)

DISCUSSION

Fusion of *S. cremoris* membrane vesicles with bR proteoliposomes by the low-pH procedure [18, 19] results in a hybrid liposome-membrane vesicle in which bR has retained its capacity to generate a proton motive force. Although the magnitude of the Δp is relatively low, the inside-out orientation of the bR molecules makes this system excellently suited for studies on solute extrusion systems. The reason that the Δp values generated in the fused membranes are relatively low is most likely the result of a highly passive ion premeability. Recently we described a model system for transport studies in membranes with a Δp, interior negative and alkaline. This system was obtained by fusion of membrane vesicles of *S. cremoris* with cytochrome *c* oxidase containing proteoliposomes [36]. Since the turnover of cytochrome *c* oxidase is about 200-fold higher than the turnover of bacteriorhodopsin (A. J. M. Driessen, unpublished results), a high Δp can be generated in this model system.

Despite the low Δp values generated by bR upon illumination of the fused membranes, the studies with ionophores clearly demonstrate that the ΔpH, interior acid, functions as a driving force for Ca^{2+} uptake. Ca^{2+} uptake in *S. cremoris* membrane vesicles is most likely catalyzed by a Ca^{2+}/H^+ antiport system and is therefore a secondary transport process. This conclusion is confirmed by the observation that an artificially imposed nigericin-mediated potassium diffusion gradient which leads to an acidification of the vesicle interior is sufficient to drive Ca^{2+} accumulation. The initial Ca^{2+} uptake rate was shown to be dependent on the magnitude of the nigericin-mediated potassium diffusion gradient. Furthermore Ca^{2+} efflux from whole cells and membrane vesicles resulted into an acidification of the internal volume as shown by the accumulation of methylamine. In *S. faecalis* calcium extrusion is mediated by a Ca^{2+} ATPase [6]. Heefner and Harold [37] showed that sodium extrusion from intact *S. faecalis* cells was a primary transport process while uptake by everted membrane vesicles could be either primary or secondary. These authors suggested that a primary sodium ATPase could be converted into a secondary sodium/proton exchange by proteolysis, although recent studies indicated that the primary sodium ATPase catalyzes sodium/potassium exchange [16]. Our studies with intact cells demonstrate that the calcium/proton exchange activity of *S. cremoris* membrane vesicles are not due to a proteolytic artefact. However, an unambiguous interpretation of the action of vanadate on Ca^{2+} transport in whole cells is not yet possible. Since phosphate and vanadate are likely to be transported by a common transport system [38], high concentrations of phosphate can prevent the uptake of vanadate and thus the inhibitory action. Furthermore, high concentrations of phosphate which can compete with vanadate are present in energy-starved *S. cremoris* [39]. Finally, a reduction of vanadate to the inactive vanadyl could occur in cells, just as has been observed in erythrocytes [40]. Ca^{2+} extrusion is

highly sensitive towards $(cHxN)_2C$, which blocks the H^+-ATPase and therefore Δp generation. Although not shown, 100 μM $(cHxN)_2C$ completely abolished lactose induced $\Delta\psi$ generation in whole cells. Since $(cHxN)_2C$ did not affect Ca^{2+} uptake in membrane vesicles (Table 2) energized by means of a nigericin-catalyzed potassium diffusion gradient, the inhibitory effect of $(cHxN)_2C$ cannot be attributed to a direct inhibition of the antiport as observed for the Ca^{2+}/H^+ antiport of *E. coli* [8]. The secondary nature of the Ca^{2+} extrusion system of *S. cremoris* is further supported by the observation that ATP driven Ca^{2+} uptake by french press membrane vesicles of *S. cremoris*, which are presumably inside-out oriented, is sensitive towards uncouplers (A. J. M. Driessen, unpublished results). Ca^{2+} uptake by *S. cremoris* membrane vesicles is strongly dependent on external phosphate which either suggests that Ca^{2+} is accumulated in symport with phosphate as has been demonstrated for Ca^{2+} uptake by everted vesicles of *E. coli* [8] or is a result of a trapping function of internally precipitated calcium-phosphate complexes. A similar phosphate dependence has been reported for the electrogenic Ca^{2+} uniporter in *Bacillus subtilis* membrane vesicles [5]. Since Ca^{2+} efflux-induced proton fluxes did not appear to be dependent on external phosphate, the phosphate dependence of Ca^{2+} uptake is most likely a result of a trapping function of the anion. In contrast to studies with membrane vesicles, Ca^{2+} efflux from intact cells appeared to be electrogenic. Valinomycin inhibited Ca^{2+} efflux at pH 7.5 and stimulated Ca^{2+} efflux at pH 5.5. Since bR generates a ΔpH of about 0.7 in the presence of nonactine, the internal pH of the fused membranes is below pH 7.0 which might explain the apparent electroneutral Ca^{2+}/H^+ exchange. No further experiments were performed at a pH above 7.5 since energy-independent Ca^{2+} binding increased dramatically above this pH. Furthermore bR became highly susceptible to denaturation at alkaline pH values.

The results presented in this paper indicate that Ca^{2+} uptake in *S. cremoris* membrane vesicles is a secondary transport process and is catalyzed by a Ca^{2+}/H^+ antiport system.

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